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ORIGINAL PAPER

Effect of rhizobial inoculations on nitrogen metabolism of *Albizia lebbek* seedlings

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Abstract: Rhizobia were isolated from *Albizia lebbek* (L) Benth. seedlings collected from six different places, tested against the nodulation test and inoculated into 45 day old *Albizia lebbek* seedlings in sterile soil mixture under glass house conditions. After a period of two and three months, the plant samples were taken to study the influence of inoculation treatments on nitrogen fixation, assimilation, biomass production and nitrogen content in different plant parts. The seedlings inoculated with isolates III(1) and III(2) of Lalpani had the maximum nodular biomass, specific (13.73 and 13.59 μ mole C_2H_2 reduced h^{-1}, respectively) and total nitrogenase (11.80 and 11.16 μ mole C_2H_2 reduced h^{-1}, respectively) activities in their nodules statistically at par with each other. These also exhibited high nitrate reductase activity in different plant parts. The seedlings inoculated with slow growing isolates viz. I(2) and IV(1) and the control were amongst poor performers for biomass production, nitrogenase activity and nitrate reductase activity in different plant parts. The minimum nitrogenase (specific and total) activities and low nitrogen content (%) in leaves, stems and roots were estimated in seedlings inoculated with isolate II(4) of Barkot. Nodular biomass was recorded as an indicator of nitrogen fixation activity rather than the number of nodules per plant. The isolates III(1) and III(2) can be utilized to enhance productivity in afforestation and reforestation programmes.

Key words: *Rhizobium*; biomass; nitrogenase; nitrate reductase (NR); nitrogen

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Introduction

Legumes are the most important plant groups in symbiotic nitrogen fixation (Allen and Allen 1981; Havelka et al. 1982) and second only to Gramineae in their importance to humans (Graham and Vance 2003). The nitrogen fixing ability of legumes increases the fertility of impoverished soils and enables legumes to establish and grow in ecosystem that are unsuited for most other plant groups (National Academy of Science 1979). Many woody legumes grow rapidly and serve as renewable sources of fuel, nitrogen rich green manure, high protein forage and other wood products. Due to these reasons importance of leguminous species is consistently increasing in forestry, agroforestry and wastelands reclamation programmes (Sane 1987; Vance 2001). *Albizia lebbek* (L) Benth is a fast growing multipurpose leguminous tree capable of adequate nitrogen fixation and assimilation (Pokhriyal et al. 1987; Siddiqui 1989; Prasad et al. 1997). It contributes up to 224.10 $kg \cdot ha^{-1} \cdot a^{-1}$ accretion of nitrogen into soil (Singh and Pokhriyal 1998). It can grow in a wide range of temperature, rainfall and elevation. Pokhriyal et al. (1987) reported greater plant height, nodular biomass and nitrogenase activity in *A. lebbek* seedlings when compared with *Acacia nilotica* and *Dalbergia sissoo*. *A. lebbek* nitrogenase activity peaks during the rainy season and nodular numbers and biomass peak in October (Pokhriyal et al. 1996). Like most other legumes, it can obtain much of its nitrogen requirement through symbiotic nitrogen fixation if the root nodules are formed by the effective rhizobial strains and the environmental conditions are favorable (Somasegaran and Bohlool 1990). Sufficient population of effective *Rhizobium* in soil is a critical factor in such situations (Weaver and Fredrick 1974; Roskoski et al. 1986; Singleton et al. 1992). The inoculation of *Rhizobium* in *Albizia procera*, *A. lebbek* and *Leucaena leucocephala* showed higher productivity, nodulation and nitrogenase activity as compared to controls (Aryal et al. 1999). Chauhan and Pokhriyal (2001, 2002) also reported an increase in the overall growth and nitrogen fixation potential of *A. lebbek* seedlings inoculated with *Rhizobium* and arbuscular mycorrhiza. In the

present study we investigated the relative potential of *Rhizobium* isolates from different places with respect to nitrogen fixation and assimilation in *A. lebbek* seedlings. Our results can be utilized in plantation management of this multipurpose species.

Material and methods

Isolation and inoculation of rhizobia

The seedlings of 7- to 8-month-old *Albizia lebbek* in polybags were collected from six different Forest Department nurseries from widely distributed geographical area, viz. Dehra Dun (I), Barkot (II) and Lalpani (III) in Dehra Dun Forest Division, Kalsi (IV) in Chakrata Forest Division and Kiratpur Sahib (V) in Roopnagar (Punjab) Forest Division and Lalkuan (VI) in Haldwani Forest Division (Table 1). Healthy nodules from these seedlings were collected in different test tubes, washed with water and surface sterilized in mercuric chloride (0.05% for 20 seconds). These were crushed in sterile water and streaked on *Rhizobium* medium (HiMedia M408) plates having Congo red dye. The isolated colonies, which did not absorb the color of the dye after 2 days, were picked and restreaked until the purelines were obtained. The isolated rhizobia were screened and thinned for better colonial features and tested against the nodulation test. Seeds of *A. lebbek* from a single tree source were scarified, surface sterilized and allowed to germinate aseptically in a seed germinator (Seed Buro Equipment Company, Chicago) at 30°C and 90% humidity. These were then transplanted in 2 kg pots containing autoclaved (120°C, 1 h) soil mixture (soil: farmyard manure: sand in 1:1:1 ratio) having pH 7.1±0.02 (standard error) and nitrogen, phosphorous and potassium content (%) of 0.21, 0.18 and 0.45, respectively. Pots were kept covered with a piece of clean polysheet and holes made on the cover of the pots above the seedlings to allow them to emerge without soil contamination. The rhizobial isolates were multiplied in Yeast Extract Mannitol (YEM) broth (Vincent 1970) for 12 days and 50 ml of each culture broth was inoculated into 45-day seedlings. Some of the pots were left un-inoculated to act as control.

Table 1. Details of the collection sites of isolated rhizobia

S. No.	Source place of Rhizobia	Forest Division	Latitude	Longitude	Nitrogen content (%) in soil
I	Dehra Dun	Dehra Dun	30°20’40’’N	77°52’12’’E	0.15
II	Barkot	Dehra Dun	30°49’00’’N	78°12’00’’E	0.13
III	Lalpani	Dehra Dun	30°04’13’’N	78°15’28’’E	0.13
IV	Kalsi	Chakrata	30°31’04’’N	77°50’42’’E	0.11
V	Kiratpur Sahib	Roopnagar	31°11’13’’N	76°33’51’’E	0.04
VI	Lalkuan	Haldwani	29°12’55’’N	79°32’00’’E	0.18

Description of experiment

The experiment was laid out in completely randomized design with 14 treatments including control and ten plants in each treatment. The pots were irrigated with autoclaved water and maintained under glass house conditions. After two months of inoculation in September, three seedlings per inoculation treatment were randomly selected for the study. Tap root length (cm), shoot length (cm), collar diameter (mm), number of leaves and nodules per plant were recorded. For biomass estimation, different plant parts namely leaves, stem, root and nodules were separated and oven-dried at 70°C for 72 h. Nitrogenase activity in the nodules was estimated using methods given by Hardy et al. (1968). *In-vivo* nitrate reductase (NR) was assayed in leaves, stems and roots as described by Klepper et al. (1971) with some modification (Kaur et al. 2005). The studies were repeated one month later in October. Total nitrogen was estimated in plant and soil samples taken during October using methods described by Loomis and Shull (1937). The data were analyzed using analysis of variance (ANOVA) for two factors, inoculation treatment and months; and for one factor for nitrogen content. The data pertaining to counts, viz. number of leaves and nodules, were subjected to square root transformation while percentage data i.e. nitrogen content was subjected to arc $\sin^{-1}$ transformation before applying ANOVA. Means were compared using critical difference (Scheffe 1959).

Results and discussion

Thirteen of 31 rhizobial isolates were selected and inoculated to assess their effects on nitrogen fixation, assimilation and biomass production in *A. lebbek* seedlings during the optimum period of nodulation. The meteorological data (as per instructions from the Indian Meteorological Department, Pune, India) for the duration of the experiment are given in Table 2. Significant reductions in root and shoot length ratios and increases in collar diameter and number of leaves (Table 3) and nodules (Fig. 1a) were observed from September to October. The differences between the treatments were non-significant or these parameters except the number of nodules per seedlings where the differences between treatments and month × treatment interactions were highly significant ($p \leq 0.001$). On average, the maximum number of nodules was obtained in seedlings inoculated with isolate III(1) of Lalpani followed closely by those inoculated with isolate IV(1) of Kalsi, statistically at par with each other. But the latter was inefficient in terms of nitrogen fixation. No nodules and consequently no nitrogenase activity were recorded for controls. Highly significant increases in all dry weight parameters were observed from September to October (Table 3). The inoculation treatments had significant effects ($p \leq 0.05$) on leaf and stem (Table 3) and total dry weight (Fig. 1b). Their maximum values were recorded in the seedlings inoculated with isolate II(4). The seedlings inoculated with isolates I(2) and IV(1) from Dehra Dun and Kiratpur Sahib, respectively, alongwith controls were amongst the poor biomass producers. These two isolates were also found to grow slowly under *in-vitro* conditions. Umali-Garcia et al. (1988) reported that rhizobial isolates influenced the growth and nodulation in *Albizia falcataria* and *Acacia maginum*. Dela-Cruz et al. (1988) reported poor growth response in *Acacia mangium* seedlings inoculated with a VAM + *Rhizobium* or *Rhizobium* alone as compared to

un-inoculated controls. Poor growth response was reported in *Acacia nilotica* inoculated with *Rhizobium* or *Rhizobium* + VAM (Thapar et al. 1996).

Table 2. Meteorological data at New Forest Campus, Forest Research Institute, Dehra Dun for the duration of the experiment

Month	Temperature (°C)			Vapour Pressure (mm Hg)		Relative Humidity (%)		Rainfall	Bright Sunshine	No. of
	Max.	Min.	Mean	07.19 hr	14.19 hr	07.19 hr	14.19 hr	(mm)	Hr/day	rainy days
May	33.7	19.4	25.8	17.5	17.5	79	48	144.3	7.8	10
June	31.1	21.5	25.5	20	20.2	87	67	292.1	5.9	11
July	29.9	22.8	25.7	21.5	22.1	92	78	701.5	3.4	20
August	30.1	22.2	25.5	21.1	22.3	93	76	560.9	4.1	20
September	29.7	19.6	24.1	18.4	19.4	94	68	241.6	7.1	10
October	29.6	14.5	21.7	13.6	16.3	95	56	0	8.1	0

Table 3. Effect of rhizobial inoculations on various growth and biomass parameters in *A. lebbek* seedlings under controlled conditions

Inoculation treatment	Tap root length: shoot length ratio			Collar diameter (mm)			Number of leaves			Leaf dry weight (g)			Stem dry weight (g)			Root dry weight (g)			Nodule dry weight (g)		
	Sept	Oct	Mean	Sept	Oct	Mean	Sept	Oct	Mean	Sept	Oct	Mean	Sept	Oct	Mean	Sept	Oct	Mean	Sept	Oct	Mean
Control	0.85	0.31	0.58	2.81	5.85	4.33	22 (4.69)	25.67 (5.07)	23.83 (4.88)	1.9	2.98	2.44	1.45	3.68	2.57	1.9	5.24	3.57	0	0	0
I(1)	0.96	0.3	0.63	3.35	5.16	4.25	21.33 (4.62)	28.67 (5.35)	25 (5)	2.08	3.03	2.56	1.47	3.73	2.6	3.54	5.87	4.71	0.01	0.11	0.06
I(2)	0.65	0.57	0.61	2.68	4.41	3.54	22.67 (4.76)	25.33 (5.03)	24 (4.9)	2.14	2.56	2.35	1.58	2.2	1.89	1.77	5.75	3.76	0.1	0.03	0.06
II(1)	0.97	0.35	0.66	2.96	5.01	3.98	23 (4.8)	33.33 (5.77)	28.167 (5.31)	2.25	3.27	2.76	1.5	3.51	2.51	2.27	5.5	3.88	0.02	0.05	0.03
II(2)	1.04	0.44	0.74	2.51	4.93	3.72	22.67 (4.76)	30.67 (5.54)	26.67 (5.16)	2.13	2.89	2.51	1.21	3.35	2.28	1.92	5.97	3.95	0.03	0.02	0.02
II(3)	0.84	0.38	0.61	3.19	6.27	4.73	28 (5.29)	31.33 (5.6)	29.67 (5.45)	1.94	3.06	2.5	1.58	4.95	3.26	2.41	6.04	4.23	0.03	0.13	0.08
II(4)	0.89	0.44	0.67	3.37	6.77	5.07	24.67 (4.97)	32.33 (5.69)	28.5 (5.34)	2.77	3.39	3.08	1.62	5	3.31	2.73	7.42	5.08	0.01	0.13	0.07
III(1)	0.85	0.38	0.62	3.55	5.89	4.72	25.33 (5.03)	31 (5.57)	28.17 (5.31)	2.34	2.87	2.6	1.69	3.44	2.57	2.47	5.85	4.16	0.15	0.16	0.15
III(2)	0.8	0.37	0.59	3.61	5.37	4.49	18.33 (4.28)	34 (5.83)	26.17 (5.12)	2.65	2.85	2.75	2.48	3.16	2.82	2.47	6.36	4.42	0.05	0.26	0.15
IV(1)	0.94	0.5	0.72	3.11	5.87	4.49	24.67 (4.97)	25 (5)	24.83 (4.98)	1.62	2.16	1.89	1.57	2.68	2.13	1.94	6.5	4.22	0.01	0.13	0.07
IV(2)	0.64	0.43	0.54	3	5.77	4.38	26.67 (5.16)	30.67 (5.54)	28.67 (5.35)	2.15	2.91	2.53	1.9	3.45	2.68	2.63	7.12	4.87	0.01	0.2	0.1
V(1)	0.96	0.34	0.65	2.82	4.75	3.79	24 (4.9)	30.33 (5.51)	27.17 (5.21)	2.04	2.49	2.27	1.28	2.65	1.97	1.99	6.76	4.38	0.03	0.03	0.03
V(2)	0.6	0.61	0.61	3.48	5.85	4.66	22 (4.69)	32.67 (5.72)	27.33 (5.23)	2.42	3.21	2.82	2.1	3.04	2.57	2.34	7.38	4.86	0	0.12	0.06
VI(1)	1.13	0.36	0.74	2.8	5.76	4.28	27.67 (5.26)	26.33 (5.13)	27 (5.2)	1.88	2.94	2.41	1.25	4.03	2.64	1.97	5.84	3.9	0.11	0.07	0.09
Mean	0.87	0.41		3.09	5.55		23.79 (4.86)	29.81 (5.44)		2.16	2.9		1.62	3.49		2.31	6.26		0.04	0.1	

Critical difference at $p \leq 0.05$

	Tap root length: shoot length ratio	Collar diameter (mm)	Number of leaves	Leaf dry weight (g)	Stem dry weight (g)	Root dry weight (g)	Nodule dry weight (g)
Month	0.09***	0.36***	1.79***	0.22***	0.28***	0.41***	0.04**
Treatment	NS	NS	NS	0.57*	0.74**	NS	NS
Month × Treatment	NS	NS	NS	NS	1.05**	NS	NS

Note: 1. *, ** and *** reflect significant variation at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, respectively; 2. NS denotes non-significant variation at $p \leq 0.05$; 3. The values within parentheses show the square root transformed values and critical difference in such cases has been calculated on the square root transformed values.

Highly significant ($p \leq 0.001$) increase in specific and total NR activities (in leaf, stem, root and total plant) were recorded from September to October (Fig. 2 and 3a, b, c). Significant differences between treatments were also recorded for all these variables except specific root NR activity. Maximum total leaf and stem NR activities were recorded for seedlings inoculated with isolate II(3) in October (Fig. 2b and d). Also those inoculated with isolates I(1) and III(2) exhibited high total NR activities in leaf, stem, root and plant, statistically at par with each other. Seedlings inoculated with isolates IV(1) and V(1) exhibited poor NR activity in different plant parts. Significant differences in nitrogen content (%) were observed in plant parts of various treatments (Fig. 3d). Highest nitrogen content in leaf, stem and root were estimated in seedlings inoculated with isolate I(2). These seedlings had comparatively low nitrogenase activity but the NR activities in different plant parts were quite good, which may be responsible for high nitrogen content in leaf, stem and root. Earlier also, it has been reported that plant tend to complement their nitrogenase activity with NR activity in order to meet persistent nitrogen requirements from soils and the atmosphere in the case of *D. sissoo* (Pokhriyal et al. 1991) and *A. lebbek* (Kaur et al. 2005). Somasegaran et al (1990) also reported that shoot nitrogen content (%) was a poor indicator of nitrogen fixation capacity in Bombara groundnut. Soil nitrogen content differed non-significantly between various treatments (data not shown). Most probably the

time duration of the experiment was insufficient for nitrogen accretion into soil.

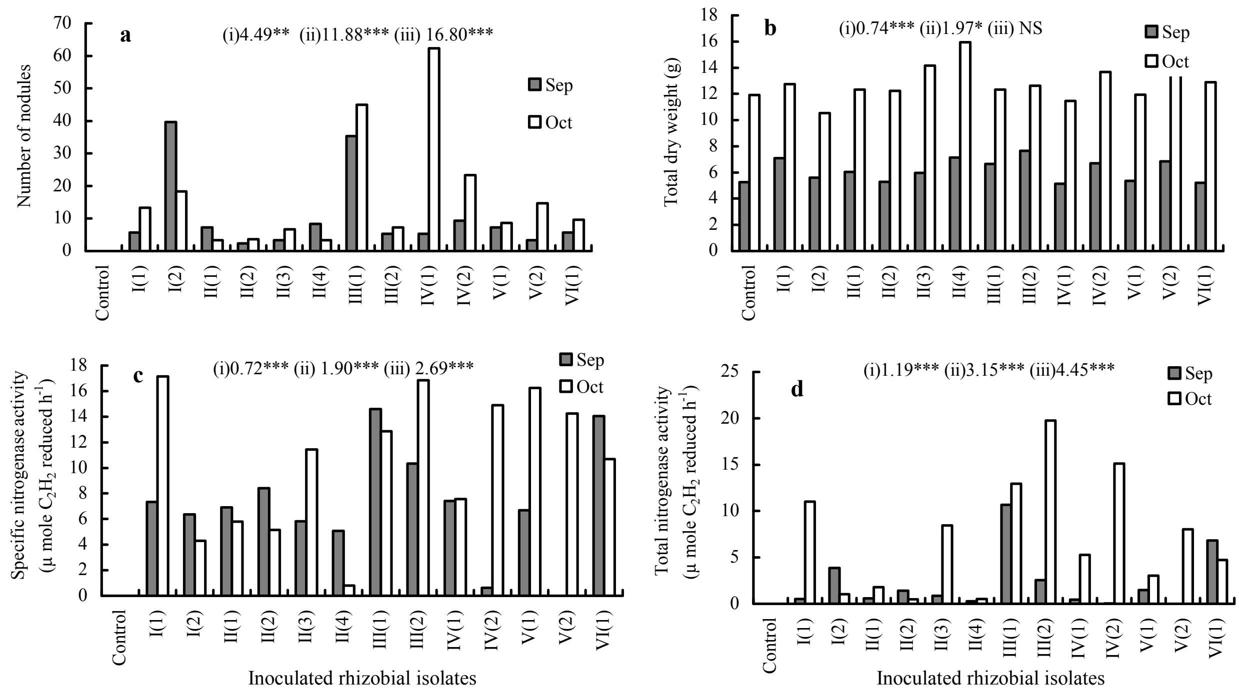

Fig. 1 Nodule number, biomass and nitrogenase activity in rhizobial inoculated *A. lebbek* seedlings under controlled conditions. Note: 1. (i), (ii) and (iii) denoted the CD values wherever the differences among months, treatments and month × treatments respectively were statistically significant. 2. *, ** and *** reflect significant variation at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively; 3. NS denotes non-significant variation at $p \leq 0.05$.

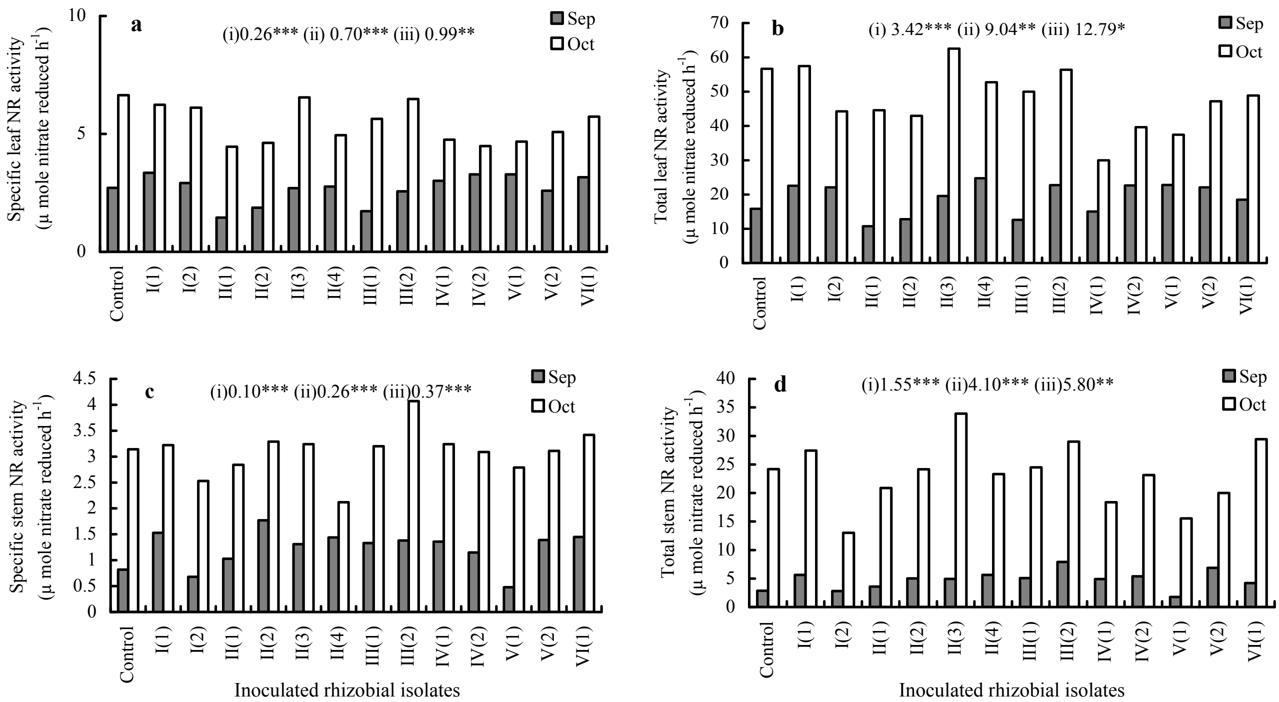

Fig. 2 Nitrate reductase activity in leaf and stem of rhizobial inoculated *A. lebbek* seedlings under controlled conditions. Note: 1. (i), (ii) and (iii) denote the CD values wherever the differences among months, treatments and month × treatments respectively were statistically significant. 2. *, ** and *** reflect significant variation at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, respectively.

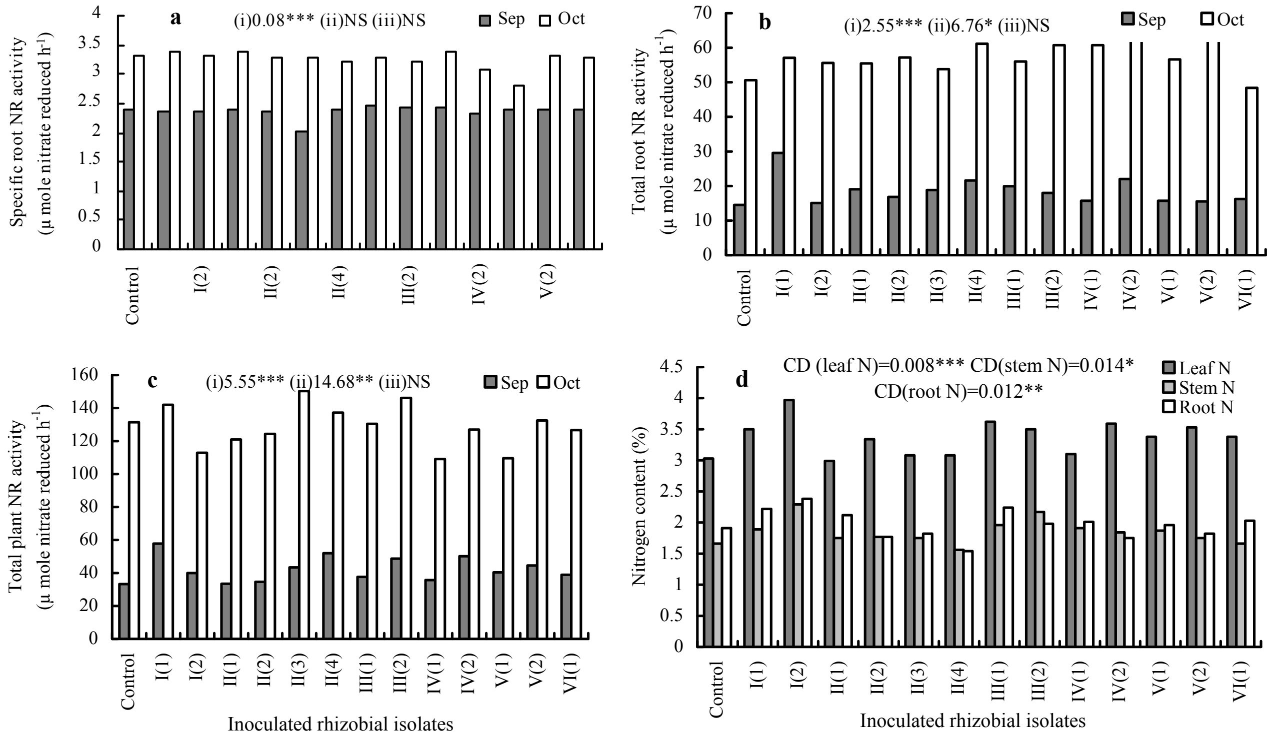

Fig. 3 Root and total plant nitrate reductase activity and nitrogen content in different plant parts in rhizobial inoculated *A. lebbek* seedlings under controlled conditions. Note: 1. (i), (ii) and (iii) denote the CD values wherever the differences among months, treatments and month × treatments respectively were statistically significant. 2. *, ** and *** reflect significant variation at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, respectively. 3. NS denotes non-significant variation at $p \leq 0.05$.

The nitrogen content of leaves, stems and roots were among the lowest for seedlings inoculated with II(4), which also exhibited minimum specific and total nitrogenase activities. This is in consonance with our earlier investigations (Kaur et al. 2005). Somasegaran et al. (1990) reported a significant influence of rhizobial isolates on shoot nitrogen content in Bombarra groundnut. Turk et al. (1993) observed statistically significant increases in shoot nitrogen content of tree legumes due to inoculation treatments in soil with inadequate rhizobial populations. Elkoca et al. (2008) reported significant increase in growth, biomass, N% and chlorophyll content after seed inoculation of chickpea with *Rhizobium*, nitrogen fixing *Bacillus subtilis* and phosphorus solubilizing *Bacillus megaterium*.

Inoculation with appropriate *Rhizobium* isolates ideally suited to the species and environment in terms of nitrogen fixation and assimilation, enhances productivity of forests and plantations. There is a possibility that the isolate II(4), which led to maximum collar diameter and biomass in *Albizia lebbek* seedlings can be exploited in plantations to be managed for fuel, fodder and timber. The isolates III(1) and III(2), which caused maximum specific and total nitrogenase activity and nodular biomass and high nitrate reductase activities can be utilized for afforestation and reforestation programmes on lands with low nitrogen content. Since our results are for pot experiments conducted under glasshouse conditions, widespread field testing is required to assess the practicability of our conclusions.

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